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A member of the chicken RXR family of nuclear receptors activates transcription in response to retinoic acid

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The chicken cRXR nuclear receptor is a member of the steroid/thyroid hormone receptor superfamily. In this paper we show that cRXR can transactivate gene expression in response to retinoic acid, but that its sensitivity to retinoic acid is lower than that of the hRAR-β receptor. We have also compared the ability of cRXR and hRAR-β to respond to a panel of other retinoids. Unlike hRAR-β, cRXR failed to respond to the naturally occurring retinoid 3,4-didehydro-retinoic acid or to the synthetic retinoid TTNPB, both of which share the ability of retinoic acid to induce digit duplications when locally applied to chick limb buds.

Retinoic acid; Retinoic acid receptor; RXR; Transcriptional control; Transactivation

1. INTRODUCTION

all-trans-Retinoic acid (RA) is a metabolite of vitamin A that affects differentiation and morphogenesis in a number of systems. For example, local application of RA to chick limb buds induces digit duplications (reviewed in [1]). RA and the related metabolite 3,4-didehydro-*all-trans*-retinoic acid (3,4-didehydro-RA) are present in chick embryos [2], and may be natural signalling substances in the limb bud (reviewed in [1]). In *Xenopus laevis* embryos, RA can affect patterning of the central nervous system [3,4] and in mammals, retinoids are potent teratogens [5,6]. In addition to their effects on embryos, retinoids affect the differentiation and maintenance of epithelial cells [7,8] and are used to treat a number of dermatoses [5].

The biological effects of retinoids are mediated at the cellular level by their interaction with cytoplasmic binding proteins and nuclear receptors. Humans and mice possess three closely-related RA receptors (RAR-α, RAR-β and RAR-γ) that belong to the steroid/thyroid hormone receptor superfamily (reviewed in [9]). Binding of RA converts the RAR into an active transcription factor that can regulate the expression of target genes by binding to RA response elements (RAREs). Amongst the genes that contain an RARE are the alcohol dehydrogenase 3 gene [10] and the RAR-β gene [11]. RARs can also regulate transcription by binding to a subset of thyroid hormone response elements (TREs), indicating that RA and thyroid hormone may regulate overlapping sets of genes [12].

A fourth nuclear receptor that can regulate gene expression in response to RA was recently discovered in humans [13]. This receptor (hRXR-α) has amino acid similarity to the RARs in the DNA-binding domain alone and so represents a distinct class of receptor. Mice possess a related receptor (mRXR-β), and there may be other RXR family members in humans and mice [13]. It has not been reported whether mRXR-β can respond to RA. Since hRXR-α is less sensitive than hRAR-α to RA, it may be that a structurally related compound, rather than RA itself, is the natural ligand of hRXR-α [13].

We recently isolated a chicken cDNA clone that encodes a protein, named cRXR, with amino acid sequence similarity to hRXR-α and mRXR-β [14]. Differences in the amino-terminal acid sequences of these three proteins suggest that cRXR may represent a third, distinct, member of the RXR family. Transcripts of the cRXR gene are present in liver, in migrating neural crest cells and in a range of neural-crest-derived cell types, including cells of the peripheral nervous system [14]. The peripheral nervous system is an unexpected site for the expression of an RA receptor [14] and so these data questioned whether cRXR would respond to RA.

In this paper, we show that cRXR is able to respond to RA, indicating that RA-responsiveness is a general property of the RXR family of nuclear receptors. Our results also show that the responsiveness of cRXR to RA and to other retinoids differs from that of hRAR-β and more closely resembles that reported for hRXR-α [13].

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2. MATERIALS AND METHODS

2.1. *Plasmids*

A *HincII–DraI* fragment of the cRXR cDNA clone pR2 (nucleotides 1–1667) [14], containing the entire cRXR coding region, was cloned into the *SmaI* site of expression vector pJ4Ω [15], placing the insert under the control of the Moloney murine leukaemia virus long terminal repeat. This yielded plasmids pcRXR and pcRXR (ant), with inserts in opposite orientations. The hRAR-β expression plasmid RAR-β0 [16] was a gift from Pierre Chambon, Strasbourg, as was the reporter plasmid $(TRE3)_3$-*tk*-CAT, which contains the chloramphenicol acetyl transferase (CAT) gene under the control of the herpes simplex virus thymidine kinase gene promoter and a synthetic TRE [17]. Plasmid pCH110 [18], which contains the *E. coli* β-galactosidase gene under the control of the SV40 promoter, was purchased from Pharmacia (Milton Keynes, UK).

2.2. *Cell culture and transfections*

C13 baby hamster kidney (BHK) cells were cultured at 37°C in 5% CO_2/air in DMEM supplemented with 4.5% glucose and 10% foetal calf serum.

Plasmid pcRXR, pcRXR (ant) or RAR-β0 DNA (5 μg) was transfected into BHK cells with 1 μg $(TRE3)_3$-*tk*-CAT DNA, 0.5 μg pCH110 DNA and 5 μg autoclaved herring testis carrier DNA, using the calcium phosphate precipitation technique [19]. The precipitate was left on the cells overnight and then replaced with fresh medium. After 48 h, transfected cultures were treated with RA, retinal, retinol, retinyl acetate, retinyl palmitate, 3,3',5-triiodo-L-thyronine, cholecalciferol, dexamethasone, pregnenolone, 25-hydroxycholesterol, β-oestradiol, dihydrotestosterone (all from Sigma, St Louis, MO), *p*-([E]-2-[5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl-6,7-propenyl)benzoic acid (TTNPB) or 3,4-didehydro-RA (both from Hoffmann-La Roche, Basel). Since stock solutions of these compounds were prepared in ethanol, equal volumes of ethanol were added to control cultures. In all cases, less than 10 μl of ethanol were added per 1 ml of culture medium.

2.3. *CAT and β-galactosidase assays*

After 48 h in the presence of ligand, cell extracts were assayed for CAT activity by thin layer chromatography (TLC), using [^{14}C]chloramphenicol [19]. TLC plates were autoradiographed, regions corresponding to autoradiographic signals were excised and the amount of radioactivity that they contained was determined by liquid scintillation counting. The percentage of total chloramphenicol converted to acetylated forms was calculated. Aliquots of the same cell extracts were assayed for β-galactosidase activity [19].

3. RESULTS AND DISCUSSION

3.1. *Activation of cRXR by retinoic acid*

Expression plasmids containing the cRXR insert in both sense (pcRXR) and antisense (pcRXR (ant)) orientations were constructed, the former to direct expression of cRXR protein and the latter to act as a negative control. Analysis of the predicted amino-acid sequence of the DNA-binding domain of cRXR [14], indicated that cRXR should be able to stimulate transcription from a promoter containing a palindromic TRE [13]. We therefore co-transfected BHK cells with $(TRE3)_3$-*tk*-CAT and either pcRXR or pcRXR (ant). As shown in Fig. 1, CAT activity in cells transfected with pcRXR increased approximately six-fold after treatment with 10^{-6} M RA. No such increase was seen in cells transfected with pcRXR (ant). This demonstrates that cRXR can activate gene expression in response to RA.

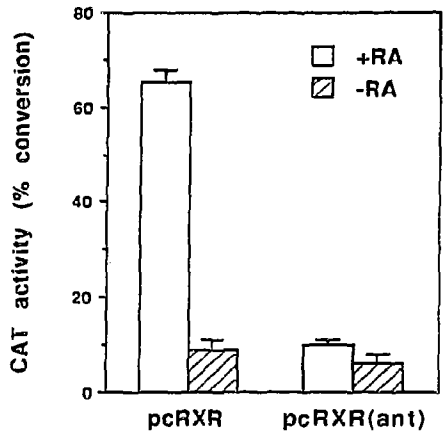

Fig. 1. RA-dependent activation of transcription by cRXR: CAT activity in cells transfected with pcRXR or pcRXR(ant) and treated with 10^{-6} M RA (+RA) or ethanol carrier (−RA). Transfection efficiency was the same for each culture, as determined by co-transfection with pCH110. Values represent the mean percentage conversion of chloramphenicol to its acetylated derivatives in three independent experiments ± SD.

3.2. *Dose–response of cRXR to retinoic acid*

We next compared the dose–responses of cRXR and hRAR-β to RA by co-transfecting BHK cells with $(TRE3)_3$-*tk*-CAT and either pcRXR or RAR-β0, and treating the transfected cells with increasing doses of RA. The maximum dose used was 10^{-5} M, since RA does not fully dissolve at higher concentrations. The dose–response curve for hRAR-β (Fig. 2b) was similar to that previously reported for RAR family members [13]. Comparison with the dose–response curve for cRXR (Fig. 2a) indicated that cRXR was less sensitive than hRAR-β to RA. Since the cRXR dose–response curve did not reach saturation it was difficult to estimate accurately the difference in sensitivity. However, the RA concentration required by cRXR to induce 50% of the maximum detected CAT activity was approximately fivefold greater than that required by hRAR-β to induce 50% maximal CAT activity. In the absence of saturation this is likely to be an underestimate of the difference in sensitivity. This resembles the case of hRXR-α, which requires at least a five-fold greater concentration of RA for 50% maximal induction of CAT activity than does hRAR-α [13].

3.3. *Response of cRXR to other retinoids*

Finally, we compared the ability of cRXR and hRAR-β to respond to a range of retinoids following co-transfection of BHK cells with $(TRE3)_3$-*tk*-CAT and either pcRXR or RAR-β0. As shown in Fig. 3, RA, retinal and TTNPB activated hRAR-β efficiently and with approximately equal potency, whilst retinol, retinyl acetate and retinyl palmitate were much less potent. A similar pattern of response has been demonstrated previously for hRAR-α, hRAR-β and hRAR-γ [13]. In contrast, whilst cRXR responded to RA and retinal,

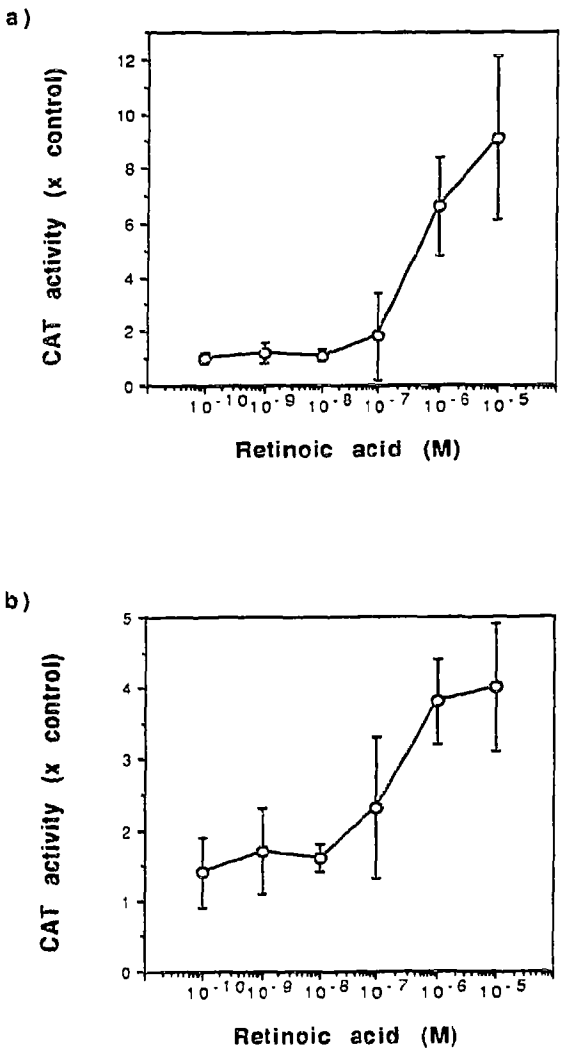

Fig. 2. Dose–response of (a) cRXR and (b) hRAR-β to RA. CAT activity was normalised for transfection efficiency by comparison with the β-galactosidase activity directed by co-transfected pCH110. Values plotted represent CAT activity in treated cells, compared to CAT activity in control cells treated with ethanol carrier, and represent the mean of three independent experiments ± SD.

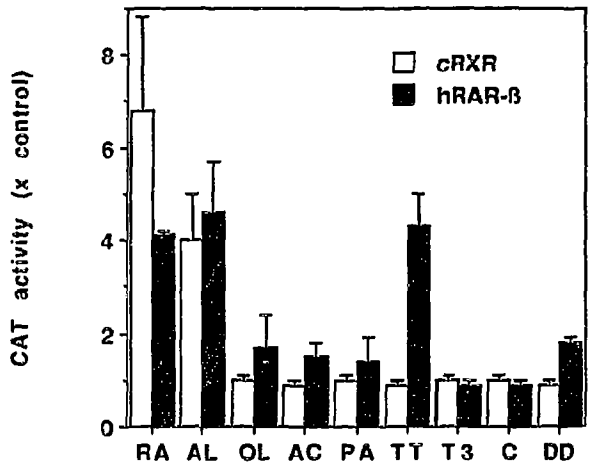

Fig. 3. Response of cRXR and hRAR-β to a range of retinoids: 10^{-6} M RA (RA); 10^{-6} M retinal (AL); 10^{-6} M retinol (OL); 10^{-6} M retinyl acetate (AC); 10^{-6} M retinyl palmitate (PA); 10^{-6} M TTNPB (TT); 10^{-6} M 3,3',5-triiodo-L-thyronine (T3); a cocktail (C) containing cholecalciferol, dexamethasone, dihydrotestosterone, 25-hydroxycholesterol, β-oestradiol and pregnenolone, each at 10^{-6} M; 10^{-7} M 3,4-didehydro-RA (DD). CAT activity was normalised for transfection efficiency by comparison with the β-galactosidase activity directed by co-transfected pCH110. Values plotted represent CAT activity in treated cells, compared to CAT activity in control cells treated with ethanol carrier, and represent the mean of two independent experiments ± SD.

there was no detectable response to retinol, retinyl acetate, retinyl palmitate or TTNPB. There was no detectable response of either cRXR or hRAR-β to 3,3',5-triiodo-L-thyronine (thyroid hormone) or to a cocktail of ligands known to activate other members of the steroid/thyroid hormone receptor superfamily. The pattern of response of cRXR resembles that of hRXR-α, which is also activated by RA and retinal but not by retinol, retinyl acetate, retinyl palmitate or TTNPB [13].

Thaller and Eichele [2] recently reported the isolation of a novel biologically active retinoid, 3,4-didehydro-RA, from chick embryos. As shown in Fig. 3, cRXR did not respond to 3,4-didehydro-RA, whilst hRAR-β did. It has not been reported whether hRXR-α responds to 3,4-didehydro-RA.

The lack of response of cRXR and hRXR-α to TTNPB or of cRXR to 3,4-didehydro-RA, is striking since both of these compounds are potent inducers of extra digits when locally applied to the chick limb bud [2,20]. This argues that the effects of retinoids on patterning in the chick limb bud are not mediated by the RXR family of nuclear receptors. In agreement with this, cRXR transcripts are not detectable in chick limb bud mesenchyme [14], although the distribution of RXR-α and RXR-β transcripts is not known. The RAR family of nuclear receptors, which do respond to TTNPB and 3,4-didehydro-RA, may mediate these effects [1].

Our data demonstrate that a member of the chicken RXR family of nuclear receptors is able to respond to RA and resembles human RXR-α in terms of its sensitivity to RA and its responsiveness to a panel of other retinoids. The conservation of retinoid-response characteristics between distinct RXR nuclear receptors from an avian and a mammalian species argues that the RA responsiveness of these molecules does not represent a spurious cross-reaction and supports the idea that the natural ligand of the RXR class of nuclear receptors is structurally related to RA. Mangelsdorf et al. [13] have suggested that another metabolite of retinol could be a higher affinity ligand. Our data exclude the possibility that the naturally occurring metabolite 3,4-didehydro-RA is such a ligand but leave open the possibility that another, as yet unidentified, metabolite of retinol is the natural ligand of the RXR class of nuclear receptors.

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